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Date: October 13, 2003

Paige A. Johnson

Attorney Docket No. 11000.1021c1U

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re : **Marion Wood et al.**
Group Art Unit: 1637
Application No. : 09/640,211
Filed : August 16, 2000
Title : **COMPOSITIONS AND METHODS FOR THE
MODIFICATION OF GENE TRANSCRIPTION**
Examiner : Joyce Tung

DECLARATION OF DR. MARION WOOD

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The undersigned hereby declares:

1. I am presently a Research Scientist at Genesis Research and Development Corporation Limited, the assignee of the subject patent application, and an inventor of the subject patent application. I have a PhD in the field of Plant Molecular Biology and Biochemistry.
2. The following experiments on the use of transcription factor DNA binding domains to modify gene expression *in planta* were carried out by myself with the assistance of other individuals working under my direct supervision.

To circumvent potential lethal phenotypes commonly observed with the ectopic constitutive expression of transcription factors *in planta*, constructs expressing the 5' terminal DNA binding domains of MYB transcription factors (MYB TF) can be generated, under the control of either a constitutive or cell-specific promoter. It is

hypothesized that ectopic expression of the truncated MYB TF creates an *in planta* competitive scenario for the endogenous MYB TFs and transgene product. Binding of this DNA binding domain product, with no associated activation domain, inhibits, or severely reduces, both the ability of the endogenous TF to bind to the appropriate *cis*-element and inhibits transcriptional activation of the downstream gene, resulting in a knock-out like phenotypic effect.

The *Eucalyptus grandis* transcription factor given in SEQ ID NO: 2076 shares amino acid sequence identity with MYB transcription factor genes from other plant species, as demonstrated by the BLAST homology searches provided in Exhibit A submitted herewith. This gene was identified from a cDNA library derived from *Eucalyptus grandis* floral buds.

Analysis of the amino acid sequence using publicly available tools such as PROSITE and InterPro, readily identified putative DNA binding domains present in SEQ ID NO: 2076 (see Jin and Martin, *Plant Mol. Biol.* 41:577-885, 1999). The amino acid sequences of the identified DNA binding domains are given in SEQ ID NO: 2346 and 2347.

The adjacent putative DNA binding domains were amplified as a single fragment by polymerase chain reaction (PCR) (Forward primer 5' CGTCTGTCTAGAAACAAGCTGAACATGGACAAGAAGC 3' and Reverse primer 5' TGGCCTTCTAGACTAGCTCTGACCAGAGAAA 3', with the aim of adding *Xba*I restriction sites on both the 5' and 3' termini to facilitate cloning using conventional restriction enzyme-based protocols, and a termination codon (TAG) to the 3' terminus only. These primers amplified nucleotides 21 to 424 of SEQ ID NO: 2076. The resulting DNA binding domains fragment was cloned into the pART7/pART27 plant binary vector system (Gleave, *Plant Mol. Biol.* 20:1203-1207, 1992) for expression *in planta* under the 35S constitutive promoter.

The binary vectors were introduced into *Arabidopsis thaliana* by *Agrobacterium tumefaciens*-mediated transformation using standard floral dip procedures, as described for *Arabidopsis thaliana* (Clough and Bent, *Plant J.* 16: 735-743, 1998).

Phenotypic analysis of the resulting T2 plant translines (Table 1 below) showed that down-regulation of MYB TF activity *in planta*, associated with the over-expression of the DNA binding domains (sense orientation), resulted in a dwarfed phenotype (approximately 40% decrease in height compared to wild type (WT) or pART27 empty vector control), with spindly inflorescence bolts. Floral development

and maturation were unaffected at the temporal level. However, evidence of floral aberrations was observed in lines displaying severe growth retardation. Gross phenotypic analysis of the other translines yielded no significant phenotypes compared to wild type or pART27 empty vector control, indicating that this observed phenotype is due to the sense expression of the DNA binding domains as hypothesized. The T2 observations were made from 15 plants from 5 independent transformation lines per construct.

Table 1

Phenotypic analysis of 35S::*E. grandis* MYB TF DNA binding domain or entire ORF

Construct	Description	Line	Plant	Plant Phenotype	Histological Phenotype	PCR data
MW2	DNA Binding Domain (Sense)	2	2	Dwarf with spindly inflorescence bolt.	Decreased number of lignified cells associated with cellular collapse	Sense orientation of DNA Binding Domains verified
MW2	DNA Binding Domain (Sense)	2	3	Dwarf with spindly inflorescence bolt.	Collapsed lignified cells	Sense orientation of DNA Binding Domains verified
MW2	DNA Binding Domain (Sense)	2	4	Dwarf with spindly inflorescence bolt.	Not tested	Sense orientation of DNA Binding Domains verified
MW2	DNA Binding Domain (Sense)	2	9	Dwarf with spindly inflorescence bolt.	Decreased number of lignified cells associated with cellular collapse	Sense orientation of DNA Binding Domains verified
MW2	DNA Binding Domain (Sense)	2	11	Dwarf with spindly inflorescence bolt.	Not tested	Sense orientation of DNA Binding Domains not verified
MW7	DNA Binding	1	7	Normal WT	No vascular	Antisense

Construct	Description	Line	Plant	Plant Phenotype	Histological Phenotype	PCR data
	Domain (Antisense)				perturbation	orientation of DNA Binding Domains verified
MW7	DNA Binding Domain (Antisense)	1	8	Normal WT	Putative ectopic lignification	Antisense orientation of DNA Binding Domains verified
MW7	DNA Binding Domain (Antisense)	1	13	Normal WT	Not tested	Antisense orientation of DNA Binding Domains verified
MW7	DNA Binding Domain (Antisense)	1	14	Normal WT	Putative ectopic lignification	Antisense orientation of DNA Binding Domains verified
pART27	Empty Vector control	na	1	Normal WT	No vascular perturbation	Kan DNA verified
pART27	Empty Vector control	na	3	Normal WT	No vascular perturbation	Kan DNA verified
pART27	Empty Vector control	na	5	Normal WT	No vascular perturbation	Kan DNA verified
pART27	Empty Vector control	na	10	Normal WT	No vascular perturbation	Kan DNA verified

Histological analysis of tissue from plants transformed with the 35S::MYB DNA binding domains (sense orientation) constructs stained with Phloroglucinol and Maules showed aberrations in the morphology and lignification of the vascular structures, with obvious xylem cell collapse when compared with the controls that were transformed with empty vector. Inflorescence bolt sections from plants transformed with the DNA binding domain of SEQ ID NO: 2076 were determined to have reduced cell population in the vascular bundle and an overall reduced gross size of the vascular bundle *per se*.

The histological data showed that introduction of the binding domains of SEQ ID NO: 2076 (in sense orientation) resulted in the perturbation of vascular structure development. Further analysis of the T3 generation of the MW2 line 2 (15 individual

development. Further analysis of the T3 generation of the MW2 line 2 (15 individual progeny) indicated that the aberrant phenotype was stably inherited, with 11 of the 15 plants analyzed exhibiting a spindly phenotype.

These studies demonstrate that the sequence of SEQ ID NO: 2076 encodes a transcription factor and that the introduction into a plant of DNA constructs containing the binding domains of SEQ ID NO: 2076 in a sense orientation may be successfully employed to modify the phenotype of the plant.

3. I, the undersigned, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false statements, and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.



Marion Wood, PhD



Date 21 October 2003

EXHIBIT A

BLAST Homology of SEQ ID NO: 2076:

BLASTX SEARCH OF MYB TF DNA CONSENSUS SEQUENCE AGAINST SWISSPROT/TREMBL (29TH NOVEMBER 2002).

P93474 Myb26. SPTREMBL	286	4e-76
P81391 MYB-related protein 305. SPTREMBL	271	2e-71
P81396 MYB-related protein 340. SPTREMBL	270	3e-71
Q9LK95 ATMYB3. SPTREMBL	269	5e-71
O22684 ATMYB3. SPTREMBL	264	2e-69
Q9SPG9 Putative transcription factor. SPTREMBL	258	9e-68
Q8LCG0 Putative transcription factor MYB24. SPTREMBL	258	9e-68
Q9AVB7 LhMyb protein. SPTREMBL	241	2e-62
Q8L8W8 Putative transcription factor. SPTREMBL	238	8e-62
Q9SSA1 Putative transcription factor. SPTREMBL	237	2e-61
AAO49418 MYB9. SPTREMBL	212	1e-53
Q39552 Cpm7. SPTREMBL	209	7e-53
Q9FGY3 Myb-related transcription factor. SPTREMBL	208	9e-53
Q39551 Cpm5. SPTREMBL	208	1e-52
Q39550 Myb-related transcription factor Cpm10 (MYB transcr...	208	1e-52
Q8S3Y9 Typical P-type R2R3 Myb protein (Fragment). SPTREMBL	208	2e-52
Q8H5F5 Putative typical P-type R2R3 Myb protein. SPTREMBL	206	6e-52
Q9ARI8 Myb transcription factor JAMyb. SPTREMBL	205	1e-51
Q8S3Y6 Typical P-type R2R3 Myb protein (Fragment). SPTREMBL	201	2e-50
Q9C9G7 Putative transcription factor. SPTREMBL	200	3e-50
Q94CJ3 Putative transcription factor MYB112. SPTREMBL	198	1e-49
Q9LDE1 Putative transcription factor MYB108 (Myb-related p...	198	2e-49
Q9FTR4 Putative myb-related transcription factor. SPTREMBL	196	7e-49
Q9FNW0 Myb-related transcription factor (cpm10), putative....	196	7e-49
Q93V35 Putative dehydration-induced myb-related protein Cp...	194	3e-48
Q94FU0 Putative transcription factor MYB116. SPTREMBL	192	6e-48
Q8H257 Myb-like transcription factor 5 (Fragment). SPTREMBL	189	7e-47
Q94JN5 Myb-like transcription factor Myb 5. SPTREMBL	189	9e-47
Q8H259 Myb-like transcription factor 2 (Fragment). SPTREMBL	189	9e-47
Q8H260 Myb-like transcription factor 5 (Fragment). SPTREMBL	189	9e-47
Q94IG1 MYB transcription factor Atmyb2 (Fragment). SPTREMBL	188	1e-46
Q39028 ATMYB2 (MYB transcription factor ATMYB2). SPTREMBL	188	1e-46
Q94CZ2 Putative Myb-like transcription factor. SPTREMBL	187	2e-46
Q94JN4 Myb-like transcription factor Myb 5. SPTREMBL	187	3e-46
Q93VZ0 MYB transcription factor Atmyb2. SPTREMBL	187	3e-46
Q94IF9 MYB transcription factor Atmyb2. SPTREMBL	187	3e-46
Q94IF6 MYB transcription factor Atmyb2. SPTREMBL	187	3e-46
Q93V46 MYB transcription factor Atmyb2. SPTREMBL	187	3e-46
Q8H258 Myb-like transcription factor 5 (Fragment). SPTREMBL	187	3e-46
Q8H261 Myb-like transcription factor 5 (Fragment). SPTREMBL	187	3e-46
Q94IF7 MYB transcription factor Atmyb2. SPTREMBL	184	2e-45
Q94IF8 MYB transcription factor Atmyb2. SPTREMBL	184	2e-45
O49020 MYB-like DNA-binding domain protein. SPTREMBL	183	3e-45
Q9FRI3 Myb-related transcription factor (cpm7), putative. ...	178	1e-43
Q9LK14 MYB-like DNA-binding domain protein. SPTREMBL	173	4e-42
Q9FN86 Myb-related transcription factor-like protein. SPTR...	173	6e-42

O50069	Putative transcription factor (R2R3-MYB transcripti...	172	7e-42
Q9T0G9	Putative myb protein. SPTREMBL	172	7e-42
P92986	Snapdragon myb protein 305 homolog. SPTREMBL	171	2e-41
Q9LX82	Myb DNA binding protein-like (Putative transcriptio...	168	1e-40
Q9LRU5	MYB-related transcription factor-like protein (Put...	166	7e-40
O22264	Putative MYB family transcription factor (At2g47460...	159	5e-38
Q9SBG6	Putative transcription factor. SPTREMBL	159	5e-38
Q8L5N7	Myb-related transcription factor V1MYBB1-2. SPTREMBL	159	9e-38
Q9FR07	P-like protein. SPTREMBL	158	1e-37
Q8L5N8	Myb-related transcription factor V1MYBB1-1. SPTREMBL	158	1e-37
Q9FR08	P2-t protein. SPTREMBL	158	2e-37
Q9FR09	P2 protein. SPTREMBL	158	2e-37
Q944N2	Myb-like transcription factor. SPTREMBL	158	2e-37
MYBP_MAIZE	Myb-related protein P. SWISSPROT	158	2e-37
Q9SPG6	Putative transcription factor (Myb-related transcri...	158	2e-37
O24579	P protein. SPTREMBL	158	2e-37
Q9LDI5	Putative transcription factor (Putative MYB family ...	157	2e-37
Q43436	Cotton DNA-binding domain mRNA (Myb-like transcript...	157	3e-37
Q9S7E3	GMMYB29A1 protein. SPTREMBL	157	4e-37
Q02993	Protein 2. SPTREMBL	156	5e-37
Q8S3Z3	Typical A-type R2R3 Myb protein (Fragment). SPTREMBL	156	6e-37
Q8H271	Myb-like transcription factor 1. SPTREMBL	156	6e-37
Q9S9K9	T22J18.19 protein (At1g22640/F12K8.1). SPTREMBL	156	6e-37
Q9SEI0	WEREWOLF (MYB transcription factor WEREWOLF (WER)/ ...	156	6e-37
Q9S7Y2	Putative DNA binding protein. SPTREMBL	155	8e-37
Q9XIU9	GmMYB29A2 protein. SPTREMBL	155	8e-37
Q43524	Transcription factor. SPTREMBL	155	8e-37
Q9LE63	Putative transcription factor (Putative Myb-related...	155	1e-36
Q8S402	Typical P-type R2R3 Myb protein (Fragment). SPTREMBL	155	1e-36
Q9LNC9	F9P14.4 protein. SPTREMBL	154	2e-36
Q39153	MYB-related protein. SPTREMBL	154	2e-36
Q9FJP2	Transcription factor-like protein. SPTREMBL	154	2e-36
Q9SLT2	Myb-related transcription factor LBM1. SPTREMBL	153	3e-36
Q40174	THM18 protein. SPTREMBL	153	4e-36
BAC64999	Putative myb-related protein. SPTREMBL	153	4e-36
Q9SLT0	Myb-related transcription factor LBM4. SPTREMBL	153	4e-36
Q8LRU4	Werewolf (Fragment). SPTREMBL	153	5e-36
Q8H270	Myb-like transcription factor 1 (Fragment). SPTREMBL	153	5e-36
AAO50618	Putative myb family transcription factor. SPTREMBL	153	5e-36
AAO42191	Putative myb family transcription factor. SPTREMBL	153	5e-36
Q38851	DNA-binding protein. SPTREMBL	153	5e-36
Q9XEP0	Gibberellin MYB transcription factor. SPTREMBL	152	7e-36
Q9ATD4	GHMYB25. SPTREMBL	152	7e-36
Q9FJ07	Similarity to myb-related transcription factor (Put...	152	9e-36
Q9LHY6	EST AU082058(C12976) corresponds to a region of the...	152	1e-35
Q9LFE1	Putative transcription factor (MYB9). SPTREMBL	152	1e-35
Q8S3Y4	Typical P-type R2R3 Myb protein (Fragment). SPTREMBL	152	1e-35
Q9SDS8	Putative transcription factor. SPTREMBL	152	1e-35
Q02994	Protein 3. SPTREMBL	152	1e-35
Q9LTC4	Myb-related transcription factor. SPTREMBL	152	1e-35
O49744	R2R3-MYB transcription factor. SPTREMBL	152	1e-35
Q8SA38	Myb-related transcription factor. SPTREMBL	152	1e-35
P93417	Transcription factor GAMyb. SPTREMBL	152	1e-35
AAO42396	Putative myb family transcription factor. SPTREMBL	151	2e-35

P93474 Myb26. SPTREMBL Length = 217
 Score = 286 bits (724), Expect = 4e-76
 Identities = 150/195 (76%), Positives = 164/195 (83%), Gaps = 28/195 (14%)
 Frame = +1

Query: 31 MDKKPDDDSGKSQDVEVRKGPWTMEEDLILINYIANHGEWSNSLAKAAGLKRTGKSCRL 210
 MDKKP SQD EVRKGPWTMEEDLILINYIANHGEG WNSLAKAAGLKRTGKSCRL
 Sbjct: 1 MDKKP---CNSSQDPEVRKGPWTMEEDLILINYIANHGEVWNSLAKAAGLKRTGKSCRL 57

Query: 211 RWLNYLRPDVRRGNITTEEQLLIMELHAKWGNRWSKIAKHLPGRTDNEIKNFWRTRIQKH 390
 RWLNYLRPDVRRGNIT EEQLLIMELH+KWGNRWSKIAKHLPGRTDNEIKNFWRTRIQKH
 Sbjct: 58 RWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGRTDNEIKNFWRTRIQKH 117

Query: 391 IKQAEAFSGQ-----SSEMSD-----QASTSHMSSMPEPMETYDSPPSFQGNNNMEP 531
 IKQ + + Q S E++D Q S+S +S++ EPMETY SP S+QG +EP
 Sbjct: 118 IKQVDNPNQQNFQQKMSLEINDHHHHHPHQPSSSQVSNLVEPMETY-SPTS YQG--TLEP 174

Query: 532 LPVNLSV-----ESNEAYWSMDDLWSMQLLNGD 615
 P +N YWSM+D+WSMQLLNGD
 Sbjct: 175 FPTQFPTINNDHHQNSNCCANDNNNNNYWSMEDIWSMQLLNGD 217